In vitro maturation of ovine oocytes using different maturation media: effect of human menopausal serum

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Abstract

Objective To compare the effect of human menopausal serum with estrous sheep serum, estrous goat serum, ovine follicular fluid and bovine follicular fluid on in vitro maturation, in vitro fertilization and embryo development of sheep oocytes

Method (s) Oocytes were treated in culture with different sera and follicular fluids supplemented media to examine effects on embryo development.

Results Basic culture medium supplemented with human menopausal serum, estrous sheep serum and estrous goat serum supported better rates of *in vitro* maturation, *in vitro* fertilization and embryo development. Ovine follicular fluid and bovine follicular fluid supplementations supported similar rates of *In vitro* maturation, *In vitro* fertilization and embryo development which were lower than those supported by human menopausal serum, estrous sheep serum, estrous goat serum and control medium.

Capsule Media Supplemented with human menopausal serum, estrous sheep serum and estrous goat serum supported better rates of in vitro maturation, in vitro fertilization and embryo development.

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Conclusion Human menopausal serum, estrous sheep serum, and estrous goat serum resulted in higher maturation, fertilization and embryo development than ovine follicular fluid, bovine follicular fluid and control media.

Keywords Human menopausal serum · Ovine · Oocyte maturation

Introduction

During in vitro maturation (IVM), oocytes undergo a series of cytoplasmic changes before the resumption of nuclear maturation, leading to variable competence of the resulting embryo. The alteration of basic maturation conditions can significantly affect oocyte competence as reflected by the morula and blastocyst yield after in vitro fertilization (IVF) [1]. Consistently, successful and reliable oocyte maturation (both cytoplasmic and nuclear maturation) would dramatically improve the efficiency of preimplantation embryonic development as well as fetal development [2]. Numerous studies have examined the ability of mammalian oocytes and embryos to develop in vitro using a wide variety of culture media [3–6]. In most of the studies, the basic medium was supplemented with hormones and different concentration of sera. Sera are usually added to the IVM medium to the rate of 10–20% and are known to contain hormones, trace elements, growth factors [7], chelate heavy metal cations and serves as an osmolyte, as well as surfactant [8]. In all the experiments, maturation medium contained, bovine follicular fluid (BFF) [9], estrous goat serum (EGS) [10, 11], estrous sheep serum (ESS) [11, 12], estrous cow serum (ECS) [2], fetal bovine serum (FBS) [11, 13] and bovine serum albumin (BSA) [14] as protein supplements. The maturation medium and

the selection of protein supplements and gonadotrophins for IVM play an important role in subsequent IVF and in vitro development [6].

Exogenous gonadotrophins were found to be increased both, the number of oocytes reaching metaphase II and the overall total yield of viable embryos [15]. Gonadotrophins alter the metabolism of the cumulus cells and induce resumption of meiosis in the oocytes [16] by interrupting the mode of inhibitory substance through the gap junctions [17]. Addition of Luteinizing hormone (LH) for bovine in vitro maturation enhanced the quality of oocytes, which was reflected in an increased embryo yield after IVF and IVC. Follicle stimulation hormone (FSH) was found to enhance early embryo development rather than meiotic maturation [18]. Plasma basal levels of the gonadotrophins for individual ewes were generally <1 ng LH/mL and 20 to 60 ng FSH/mL. However, there was a surge of both LH and FSH around the time of estrus, which presumably triggers ovulation [19].

On the other hand, in women, the levels of FSH and LH will be higher if menopause has occurred. The available literature does not offer a ready comparison of the beneficial effects of human menopausal serum (HMS), ESS, EGS, BFF and ovine follicular fluid (OFF) on in vitro maturation of sheep oocytes. The present study was therefore undertaken to compare the effect of HMS with ESS, EGS, BFF and OFF on the maturation, fertilization and subsequent embryonic development of sheep oocytes.

Materials and methods

Chemicals

All chemicals were purchased from Sigma (USA), and plastics were purchased from Falcon (Paignton, U.K.) unless otherwise indicated. Except media with FSH or LH, all other solutions were filter sterilized (0.22 µm sterilizing filter. Sartorius, Germany) prior to use. All media were incubated at 39°C under a humidified atmosphere of 5% CO₂ in air for 1 h prior to use.

This study was approved by the Institutional Animal Care and Use Committee (IACUC) at Razi University.

Collection and preparation of biological materials

Blood from jugular vein of ewes and goats on the first day of estrus was collected in sterile glass tube and was allowed to clot for 2 h at room temperature (25°C). The sera were centrifuged at 1000 rpm for 10 min and then filtered with Millipore filters (Millipore Bre Co.) with the pore size of 0.22 µm. Human menopausal serum was prepared from three female donors (aged 50, 54 and 60) and pooled.

All sera were heat-inactivated at 56°C for 30 min, divided into 1 mL aliquots and kept at −20°C until they were used. Ovine and bovine follicular fluids were aspirated from transparent and healthy looking medium-size (5–15 mm) ovarian follicles. Pooled follicular fluid samples were centrifuged at 2000 rpm for 10 min. Supernatant was removed and re-centrifuged as above. Finally, the supernatant was filtered and stored at −20°C. On the day of use, the follicular fluids were heated for 30 min at 56°C in a water bath to inactivate the complement.

In vitro maturation (IVM)

Ovaries were collected from ewes of Sanjabi breeds at slaughter regardless the stage of estrous cycles and transported in thermos flask containing pre-sterilized phosphate buffered saline (PBS; Oxide, Code BR 14a, Unipath Ltd., England) supplemented with 1000 IU/mL penicillin G and 1000 mg/mL streptomycin sulfate, held at 30–34°C and returned to the laboratory within 2 h of animal slaughter. HEPES buffered tissue culture medium 199 (TCM-199) supplemented with 10% FCS, 25 IU/mL heparin and 50 µg/mL of gentamycin sulfate was used for handling oocytes prior to and after culture. Cumulus oocyte complexes (COCs) recovered from surface visible follicles using 22 G needle attached to a disposable 5 ml syringe containing 1 mL of oocyte handling medium. The COCs were isolated under a stereo zoom microscope (Nikon Corporation, Japan) and graded as good, fair or poor [20]. Only good or fair oocytes were considered acceptable and used in IVM experiments. Selected oocytes were washed three times in TMC-199. Immature ovine oocytes were matured in six different protein supplements condition. Tissue culture medium 199 (TCM-199, with Earle's salt, L-glutamine and 25 Mm Hepes; Gibco, U.K.) supplemented with pyruvate (0.2 mM), FSH (0.5 µg/mL) (SIGMA; F-4021), LH (5 µg/mL) (SIGMA; 1-7134), penicillin (100 IU/mL) and streptomycin sulfate (100 µg/mL) was used as the basic maturation medium for all of the groups. Oocytes were matured in basic maturation medium supplemented with 10% FCS (Cat. No. 10270-106 GIBCO) as a control (treatment 1) and treatments 2,3,4,5 and 6 were supplemented with 20% (v/v) HMS, ESS, EGS, OFF and BFF, respectively. After 24 h cultures, oocytes were fixed in ethanol-acetic acid (3:1), stained with 1% orcein and examined under a phase contrast microscopy (X 400). Maturation has been assumed due to the presence of metaphase II chromosome with the first polar body.

In vitro fertilization

After a 24 h incubation period, matured oocytes were partially denuded by 0.1% hyaluronidase, passing them

through a fine pipette, and then groups of 10 oocytes were transferred into 50 μ L fertilization drops covered with mineral oil. The fertilization medium was glucose-free TALP supplemented with Na pyruvate (0.2 mM), fatty- acid-free BSA (BSA-FAF, 6 mg/mL), penicillin (100 IU/mL) and streptomycin (100 μ g/mL). An epididymal spermatozoon collected from post mortem rams was washed twice with TALP sperm medium [21] containing 10% (v/v) ESS and spermatozoa lightly packed by centrifuging at 800 rpm (250 g) for 10 min. Washed sperm pellet was resuspended with TALP containing 10% (v/v) ESS. A total of 100 μ L sperm suspension was placed in the bottom of 1.5 mL of TALP containing 10% (v/v) ESS for swimming up. The concentration of separated spermatozoa was adjusted to approximately 1.5 million/mL using TALP containing 10% (v/v) ESS. Oocytes were added to this medium containing the sperm and incubated for 20–24 h.

In vitro culture

At 24 h post insemination (hpi) cumulus-free presumptive zygotes were denuded by vortexing and washing three times in PBS, passing them through a fine pipette and transferred into 50 μ L drops of embryo culture medium known as synthetic oviduct fluid, (SOF) [22] under mineral oil (25–30 presumptive zygotes per each 50 μ L drop) in a humidified atmosphere at 5% CO₂, 5% O₂ and 90% N₂ at 39°C. The SOF medium was supplemented with pyruvate (0.2 mM), BSA-FAF (8 mg/mL), 100 $\times$ MEM (20 μ L/mL), 50 $\times$ BME (10 μ L/mL), penicillin (100 IU/mL) and streptomycin (100 μ L/mL) on the day of use. During all of the IVM, IVF and IVC steps, oocytes were maintained in a humidified atmosphere at 5% CO₂, 5% O₂ and 90% N₂ at 39°C. Cleavage rate was assessed 45 hpi and blastocyst development was recorded on days 6–8 (day 0=day of IVF).

Statistical analysis

One-way analysis of variance was conducted on the data taken from the experiment to reveal the significant differences among the treatments applied with seven replications. Least Significant Difference (LSD) method was used for further mean comparison. All the analysis was performed using MSTTC computer package.

Results

In vitro maturation rate and IVF rate of ovine oocytes cultured in six different maturation media are shown in Table 1. There were significant differences in completion of nuclear maturation (metaphase II), polar body extrusion and

cumulus expansion at 24 h post maturation in HMS, ESS and EGS treatments, compared with oocytes matured in OFF, BFF and control medium (Table 1). Maturation and fertilization rates of HMS, ESS and EGS treatments were significantly ($P<0.05$) higher than that of OFF, BFF and control medium. Similarly maturation and fertilization rates between HMS and EGS treatments were significant ($P<0.05$). However there was no significant difference between ESS and EGS treatments. Ovine follicular fluid and BFF supplementation supported similar rates of IVM (68.9% and 66.9% respectively) which were lower ($P<0.05$) than those supported by control (75.1%) medium. There was significant difference in fertilization rate of oocytes matured in BFF supplementation compared with control medium (Table 1).

Data of developmental capacity of ovine oocytes matured in six different maturation media and fertilized in vitro are shown in Table 2. The cleavage rate, assessed 45 hpi, was similar for oocytes matured in OFF, BFF and control media (52.8%, 54.5% and 56.52% respectively, Table 2). Cleavage rates were increased significantly ($P<0.05$) in oocytes matured in HMS, ESS and EGS supplementation (90.3%, 87.4% and 86.5% respectively).

The proportion of morula and blastocyst stage embryos of oocytes matured in HMS, ESS and EGS supplemented media were significantly ($P<0.05$) increased over that achieved with oocytes matured in OFF, BFF and control media (Table 2). Maturation media supplemented with two kinds of follicular fluids resulted in lower ($P<0.05$) proportion of 8-cell and morula stage embryos compared with control medium. However, there was similar proportion of oocytes reaching blastocyst stage (Table 2).

Discussion

The results of this study demonstrated that media supplemented with HMS, ESS, or EGS supported better ($P<0.05$) rates of IVM, IVF and embryo development. Similarly, maturation and fertilization rates between HMS and EGS treatments were significantly ($P<0.05$) different. However, there was no significant difference between ESS and EGS treatments (Tables 1 and 2).

Maturation in TCM-199 supplemented with FCS and hormones (control medium) allowed the maturation rate of 75.1% which is higher than the 55 to 65% observed [23, 24] after 24 h of maturation in the same media. This may be due to breed differences (Sanjabi breed were used in the present study) or a more appropriate selection of COCs before maturation. Completion of meiosis, fertilization and developmental capacity in sheep oocytes were stimulated significantly by the presence of HMS, ESS and EGS in the maturation medium, whereas OFF and BFF did not have an

Table 1 The effect of supplementing IVM media¹ with different sera and kinds of follicular fluid on the IVM and IVF of sheep oocytes

Treatment groups ^a	IVM examined (n)	NO. of oocytes matured (%)	IVF examined (n)	NO. of oocytes fertilized (%)
T1=Control	237	178 (75.1)c	178	115 (64.6)c
T2=20% HMS	314	289 (92.03)a	289	260 (89.96)a
T3=20% ESS	292	261 (89.3)ab	261	231 (88.5)ab
T4=20% EGS	216	191 (88.42)b	191	164 (85.8)b
T5=20% OFF	254	175 (68.9)d	175	104 (59.42)cd
T6=20% BFF	218	146 (66.97)d	146	88 (60.27)d

^a Human menopausal serum (HMS), Estrus sheep serum (ESS), Estrus goat serum, Ovine follicular fluid (OFF), Bovine follicular fluid (BFF)

a, b, c d: Values with different superscripts in the same column are significantly different ($P<0.05$)

1: IVM median, TCM-199+10% FCS (see text for further details)

effect. Serum is a highly complex combination of components including proteins, fatty acids, vitamins, trace elements, hormones and growth factors [7, 25]. Sakaguchi et al. [26] reported that cytoplasmic maturation of oocytes during maturation without serum supplementation might be incomplete, and some other factors (possibly some serum components) are necessary for completion of cytoplasmic maturation of ovine oocytes in the *in vitro* condition. Obviously, nuclear maturation of oocytes along with cytoplasmic maturation is important at the completion of meiotic division for success of fertilization [6]. Besides, being of nutritive value, serum nurtures the cells surrounding the oocyte rather than the oocyte itself and prevents the oocyte of the zone hardening when the oocyte is liberated from the follicular environments [20]. Several studies have demonstrated that culture medium supplemented with serum can cause morphological [27, 28] and physiological [29, 30] differences in embryos compared with those produced *in vivo* or in serum free media. These differences include increased number and size of lipid droplets [31, 32] and differences in embryo quality [33, 34].

In a recent study, different concentration of ESS (5%, 10%, 20%) were added to SOFaa medium for IVF and results demonstrated that cleavage rate improved rapidly when 5–10% ESS were added to the IVM medium [35]. Moreover, the use of serum in embryo culture is known to benefit later stages of pre implantation embryo development by improving blastocyst yield [21, 36]. Walker et al. [37] reported that sheep serum was equally effective as human serum, and both were superior to either BSA or a commercial serum replacer preparation. Additionally, Moore et al. [38] demonstrated that use of serum replacer for maturation of oocytes was not beneficial; however, serum replacer may enhance embryos culture by improving development *in vitro*. Thompson et al. [39] however, found that human serum is a very effective supplement for sheep embryo culture than sheep serum itself and that factor(s) in the serum, other than albumin, promote development. Additionally Batt et al. [40] reported that human serum is superior to FCS for the development of goat zygotes.

However, Fukui et al. [41] found little difference in the development of *in vitro* fertilized cow embryo in SOF medium supplemented with either human serum or FCS. In

Table 2 The effect of supplemented IVM media¹ with different sera and kinds of follicular fluid on subsequent *in vitro* development of IVF-derived sheep zygotes ^A

Maturation media Group supplement ^a	No. of fertilized oocytes	No. of 2-4cell embryos (%)	No. of 4-8cell embryos (%)	No. of 16 cell and morula (%)	No. of blastocyt (%)
T1=Control ¹	178	65 (56.5)c	36 (55.3)b	21 (32.3)b	12 (18.4)b
T2=20% HMS	289	235 (90.3)a	173 (73.6)a	146 (62.1)a	105 (44.7)a
T3=20% ESS	261	202 (87.4)ab	153 (75.7)a	134 (63.3)a	87 (43.07)a
T4=20% EGS	191	142 (86.5)b	114 (80. 2)a	100 (70.4)a	54 (38.02)a
T5=20% OFF	175	55 (52.8)c	24 (43.6)c	15 (27.2)c	8 (14.54)b
T6=20% BFF	146	48 (54.5)c	21 (43.7)c	10 (20.8)c	7 (14.6)b

1: IVM medium, TCM-199+10% FCS (see text for further details)

^a Human menopausal serum (HMS), Estrus sheep serum (ESS), Estrus goat serum, Ovine follicular fluid (OFF), Bovine follicular fluid (BFF)

A: Experiment was conducted in seven replicates

a, b, c: Values with different superscripts in the same column are significantly different ($P<0.05$)

our present study, the addition of HMS, ESS and EGS to the basic culture medium supplemented with FCS increased the maturation rate above that of the control medium. Therefore, we observe that HMS, ESS and EGS in conjunction with FCS and gonadotrophins usually cause synergistic enhancement of nuclear maturation and embryo development. There are factors other than albumin and fraction V contaminants present in human serum of great benefit to sheep embryo development in vitro [39]. The factor(s), which benefit embryos in culture, have yet to be elucidated. However, in the present study the positive effect of HMS, ESS and EGS on IVM, IVF and embryo development may be due to high concentration of FSH and LH in these sera. Moore and Trounson [42] demonstrated that FSH and LH administration to be helpful in achieving ovine oocyte maturation in vitro. However, no effect was recorded in the number of oocytes developing to blastocysts. Later higher concentration of LH was used and an increase of 4–30% in blastocyst development was obtained [43].

The final stages of folliculogenesis primarily depend on the availability of FSH and LH. FSH is necessary for the growth of preovulatory follicles. The last step before ovulation is the LH surge. The surge is needed for the completion of meiosis I, for the release of the oocyte, and for the transition to corpus luteum function. Because of the LH surge estrogen production is decreased in the granulosa cells and progesterone synthesis increases at the expense of androgen synthesis in the theca cells [44]. LH supports follicular growth by providing androgen substrate for the granulosa cell aromatase and triggers the resumption of oocyte maturation [45].

In the present study, OFF and BFF exert an inhibitory effect rather than stimulatory effect on the in vitro maturation, fertilization and developmental capacity of sheep oocytes. Follicular fluid was previously suspected to inhibit oocyte maturation [46–48]. This may be due to presence of oocyte maturation inhibitors, a factor that isolated from bovine follicular fluid [49] which might be expected to influence maturation process [49–53]. Moreover, Choi et al. [9] reported that the complete follicular fluid from large follicles has a greater inhibitory effect on in vitro maturation, male pronucleous formation and developmental capacity of bovine oocytes, than those derived from small follicles. Follicular maturation and the maturation of its oocytes is maintained until the oocytes resume the nuclear maturation in response to the pre-ovulatory surge of gonadotrophins. Naito et al. [54] found that the addition of FSH overcame the inhibitory effect of follicular fluid. Similarly, Downs et al. [55] reported that epidermal growth factor and FSH could overcome meiosis arrest in mice. In the present study, 20% follicular fluid from ovine and bovine medium follicles

supplemented with FSH, LH and estradiol 17 β was added to the maturation medium of sheep oocytes. The inhibitory action of follicular fluids on oocyte maturation and developmental capacity were observed in OFF and BFF. This inhibitory effect may be due to the oocyte maturation inhibitory substance(s) in medium size OFF and BFF. On the other hand, the added amount of FSH, LH and estradiol were not strong enough to block the effect of inhibitory substance(s).

In the present study, media supplemented with HMS, ESS and EGS in the presence of FCS and hormones increased cleavage and blastocyst rates compared with media containing FCS only or OFF and BFF. Our results suggest that basic culture medium supplemented with 20% HMS, ESS and EGS synergizes with FCS, LH, FSH and E₂ more efficiently than in the other treatments and yielded higher maturation, fertilization and blastocyst development rates of sheep oocytes. Thus, the sheep oocytes matured under these protocols were of better developmental rate than oocytes in the other treatment groups.

Conclusions

Basic media supplemented with HMS, ESS and EGS supported better rates of IVM, IVF and embryo development. OFF and BFF supplementations supported similar rates of IVM, IVF and embryo development which were lower than those culture supported by HMS, ESS, EGS and control media.

However, adding human serum to animals is not well appreciated rather the other way would be well appreciated.

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